Research Article

Consequential exposure to hypothermia in gestational diabetic rats induces oxidative changes in the brain of offspring

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Received: July 02, 2020; **Accepted:** July 23, 2020

ABSTRACT

Background: Prenatal stress is unique due to range of problems and can affect the embryo/fetus beginning with conception. Gestational diabetes mellitus is the concern for expectant-mothers wherein glucose intolerance with consistent hyperglycemia is a threatening factor during pregnancy. **Objectives:** In the event of multiple stressors posing their effects on intrauterine life and placenta being the target of increased sympathetic tone during gestation, there is a possibility of functional vulnerabilities that may contribute to the pathogenesis in post-natal life. Studying brain regional discrepancies in offspring might help to know the prenatal stress-induced variation in the antioxidant barrier and promoted oxidative stress. **Materials and Methods:** The changes occurring in oxidative stress indices in discrete brain regions of rat offspring born as a consequential exposure to gestational diabetes (streptozotocin induction) and cold stress (15 and 20°C) are assessed in this study. **Results:** The findings specify the involvement of cold-stress provoked induction of higher degree oxidative stress within brain compartments as evidenced by a decrease in antioxidant enzymes, namely, superoxide dismutase, catalase, glutathione peroxidase, glutathione S-transferase, and GSH as well as increase in the concentration of malondialdehyde. Results highlight the synergistic actions of stressors due to the increased generation of free radicals. Cold stress at 15°C found to cause exacerbatory actions by depleting antioxidant enzymes in diabetic subjects than the exposures made at 20°C. **Conclusion:** The findings prove that cold stress is a crucial stimulus to a fetus during gestation and acts as a trigger of oxidative stress especially in diabetic subjects and can pose an adverse impact. These changes could partly explain the increased vulnerability of prenatally stressed subjects to functional disorders including deficits in memory and cognitive processes in later life.

KEY WORDS: Gestational Diabetes; Cold Stress; Developing Brain; Oxidative Stress Indices

INTRODUCTION

Of late, gestational diabetes mellitus (GDM) is the major concern for expectant-mothers wherein glucose intolerance with consistent hyperglycemia is a threatening factor during pregnancy.[1] As per the newest research held by the University of Adelaide in Australia, the women who get pregnant during winter are at a higher risk due to gestational diabetes.[2,3] Even in other parts of the world, the incidence has seen a definite rise in recent times. The prevalence rates are between 6% and 14% in East and West Africa[4] and between 13% and 18% in South Asia. In Indian population, the incidence of GDM is 14.42% which relies on Diabetes in Pregnancy Study Group India recommendation[5] while, its awareness and possible morbid outcomes among mothers are very low (6%).[5] Existing data suggest that the hypothalamic-pituitary-adrenal axis (HPA) and its hormonal response through cortisol, are liable for critical neurodevelopmental deficits in the juvenile offspring which is transduced due to

Access this article online	
Website: http://www.ijmsph.com	Quick Response code
DOI: 10.5455/ijmsph.2020.07116202023072020	

gestational stress.[6] Similarly, chronic intermittent cold stress influences the activation of the HPA axis.[7] However, the mechanisms underlying this HPA sensitization have not been well understood. More directly, Hanssen *et al*.[8] showed the impact of cold acclimation on insulin sensitivity in type-2 diabetes subjects, an impact that was related to markedly increased basal skeletal muscle GLUT-4 translocation. In the intrauterine environment, mild hyperglycemia swayed some metabolic changes and cold-stress spawned adrenergic overload and exaggerated the development of fetuses.[9,10] Available literature also suggests alteration(s) in the stress-activated modulatory function of norepinephrine that plays a role in sensitizing the HPA response to acute stress following a period of chronic intermittent stress.

Given the adverse impacts of multiple stressors in pregnancy, some hostile aftermaths and cognitive deficits in new-born are drawn attention in recent times. In post-natal life, brain development is sensitive to environmental influences.[11] It is an organ that is sensitive to oxidative damage because of its high metabolic activity and lipid content with the limited antioxidant defense. Several studies advocated stress-mediated alterations in the level of antioxidant enzymes, reactive oxygen species, and glutathione (GSH) system.[12,13] It is been suggested that insulin resistance during pregnancy is multifactorial[14] and hyperglycemic stress during gestation affects cerebral maturation and causes abnormalities in neural correlations, which increases susceptibility to cerebral function.[15,16] Similarly, prenatal stress caused learning deficits associated with inhibition of neurogenesis,[6] or a decline in the neuronal size in the hippocampus (H) has been reported.[17] However, no literature is out there onto clarify the molecular changes occurring in oxidative stress indices in discrete brain regions of rat offspring born as a consequential exposure to multiple stressors. Given the paucity of data that integrate gestational diabetes with cold stress on the oxidative biomarkers, there is a dire need to explicit the mechanisms by which cold stress hastens the development of diabetic complications; its impact during gestation and resultant juvenile offspring thereby this study was undertaken.

MATERIALS AND METHODS

Chemicals

Streptozotocin (STZ), bovine serum albumin, and sodium pentobarbital were obtained from Sigma-Aldrich USA. 5,5'-Dithiobis-(2-nitrobenzoic acid) (DTNB), epinephrine, and 1-chloro-2,4-dinitrobenzene (CDNB) were obtained from Merck India Ltd., Mumbai. All other chemicals are of analytical grade which were procured from SD-fine chemicals and SISCO research laboratories, India.

Animals

Laboratory acclimated healthy albino rats (200–250 g) procured from Sri Raghavendra Enterprises, Bengaluru (841/PO/Bt/S/04/CPCSEA/2017-22), were inducted to a diabetic state by a single-dose intraperitoneal injection of STZ (50 mg/kg bw in 0.1 mol/l citrate buffer pH 4.5). Three days post-STZ administration, blood samples were drawn from the tail vein and glucose levels were tested (Accu-Check Active Glucometer) and rats confirmed diabetic when their fasting blood glucose levels were more than 200 mg/dL and were selected for the experimentation. These rats along with the control group allowed to breed separately (three females:one male ratio) and were examined for the vaginal plugs, wherein females confirmed positive for pregnancy were recorded as day-1 of gestation accordingly diabetic subjects was called GDM (Gestational diabetic mellitus) and control group as non-diabetic. During experimentation, the rats were allowed free access to chow and water. All experimental procedures complied with the National Institute of Nutrition, Hyderabad (Guidelines for the Care and Use of Laboratory Animals) and were approved by the Bioethics Committee of the Faculty of Zoology at Bangalore University, Bengaluru (Protocols number: DOZ/BUB/2018-19 and 402/CPSCSEA 2009-12). Every effort was made to minimize the number of animals used and their suffering.

Experimental Design

The gestational rats on day-4 were made into groups, namely, control (Group-I); cold stress (Group-II and III); and diabetes mellitus (Group-IV). The control rats were maintained at room temperature during their entire pregnancy and intermittent cold stress regimen (3 h/day) protocol as given by Dorfman *et al*.[18] was applied to Groups-II and III animals by subjecting in a cold stress chamber (Colton, India) at 15 and 20°C, respectively, during the entire pregnancy. Likewise, GDM rats were further grouped as Groups-V and VI to induce cold stress 15 and 20°C, respectively. Post-parturition, care was taken to maintain litters along the dam for a month and used for assessment.

Biochemical Analysis

The juvenile offspring (1-month old) representing form each group (n = 6) was euthanized by spinal dislocation under 1% pentobarbital sodium (0.4 mL/100 g bw) anesthesia and discrete brain regions, namely, cerebral cortex (CC), cerebellum (CB), H, medulla oblongata, and spinal cord (SC) were isolated separately and homogenized in requisite buffers. On centrifugation, the aliquots were used to determine the following biochemical parameters connected to oxidative stress.

Lipid Peroxidation

The extent of lipid peroxidation was measured by thiobarbituric acid-reactive substances as described by Niehaus and Samuelsson[19] with slight modifications. The

assay mixture containing 1.0 mL of tissue extract and 2 mL of TCA-TBA-HCl mixture (15.00% TCA: 0.37% TBA: 0.25 N HCl=1:1:1) was incubated in boiling water for 15 min. Further, the reaction mixture was cooled and centrifuged at 1000 rpm for 10 min. The absorbance of the clear supernatant was read at 535 nm using ultraviolet/Vis spectrophotometer. The amount of formation of malondialdehyde (MDA) content was measured and expressed as "μmoles/g."

Superoxide Dismutase (SOD) (EC 1.15.1.1)

SOD activity was determined using a modified epinephrine assay as given by Misra and Fridovich.[20] At alkaline pH, superoxide anion (O_2^-) causes the auto-oxidation of epinephrine to adrenochrome. One unit of SOD is defined as the amount of extract that inhibits the rate of adrenochrome formation by 50%. In the assay, the tissue homogenate was added to 2 mL reaction mixture containing 10 Ml bovine catalase (0.4 U/μL), 20μL epinephrine (5 mg/mL), and 50 mM sodium carbonate-sodium bicarbonate buffer (pH 10.2) and the changes in absorbance were recorded at 480 nm for 5 min continuously. The specific activity of SOD was expressed in "units/mg protein." Protein content was measured by following the method given by Lowry *et al*.[21]

Catalase (EC 1.11.1.6)

The catalase activity was measured following the method of Aebi[22] as described by Mittal and Flora[23] with minor modifications. Briefly, the activity was determined by measuring the decrease in absorbance at 240 nm of a reaction mixture consisting of H_2O_2 (30 mM) in phosphate buffer, (0.1 M, pH 7.0), and requisite volume of the tissue sample. The molar extinction coefficient of 43.6 M cm^{-1} was used to determine catalase activity. The specific activity was expressed as "μmoles of H_2O_2 hydrolyzed / min/mg protein."

Glutathione Peroxidase (GPx) (EC 1.11.1.9)

The GPx activity was assayed as described by Lawrence and Burk[24] following the rate of reduced nicotinamide adenine dinucleotide phosphate (NADPH) oxidation by the coupled reaction with GSH reductase. Briefly, the reaction mixture consisted of 0.3 mL of 10 mM sodium azide and 0.2 mL of 15 mM EDTA followed by the addition of 0.1 mL of 30 mM GSH, 0.1 mL of H_2O_2, and 0.1 mL of 10 mM NADPH and 0.2 mL of tissue extract and the absorbance read at 340 nm. The specific activity of GPx was determined using the extinction coefficient 6.22 mM-1cm-1 and represented as "μmoles of NADPH oxidized/min/mg protein."

Glutathione S-Transferase (GST) (EC 2.5.1.18)

The GST activity was determined by adopting the method as given by Habig *et al*.[25] The principle of assay being the ability of GST to conjugate with GSH and CDNB, and the extent of conjugation causing is proportionate to change in the absorbance. Briefly, the reaction mixture contained 0.1 mL of tissue extract and 0.1 mL of CDNB followed by the addition of 0.1 mL of GSH (30 mM) and the activity of enzyme determined at 340 nm by reading absorbance for 5 min. The GST activity was calculated using the extinction coefficient 9.6 mM^{-1} cm^{-1} of the product formed and was expressed as "μmoles GS-CDNB formed/min/mg protein."

Reduced GSH

The reduced GSH content was estimated by the method given by Ellman.[26] The reaction between GSH and DTNB leads to the formation of yellow-colored 5-thio-2 nitro benzoic acid (TNB) whose intensity was measured spectrophotometrically at a wavelength of 420 nm. The rate of TNB product formed is directly proportional to the concentration of GSH present in the sample. The GSH content was calculated using the extinction coefficient 13.6 mM^{-1} cm^{-1} of the product formed and expressed as "μmoles /min/g."

Statistics

Statistical analysis was carried out using SPSS software 20.0. One-way Analysis of Variance (ANOVA) with *post hoc* was performed for the intergroup comparisons using Duncan's Multiple Range Test ($P > 0.05$). Graphs were plotted using "Origin Pro" software 9.0.

RESULTS

Prenatal stress during gestation has a distinct effect on their offspring and the oxidative stress indices measured in discrete brain regions are projected in the following paras.

Lipid Peroxidation

The MDA content increased significantly ($P < 0.05$) in discrete brain regions of offspring born as a consequential exposure to prenatal stress such as GD and cold stress (15 and 20°C), while cold-stress alone found to cause mild effect within the CC [Figure 1a]. In coexposure groups, cold-stress (15°C and 20°C) found to cause modulatory effect by augmenting higher MDA levels within the GD group. Among discrete brain regions, the CC, H, and medulla showed higher vulnerability to cold-stress and as compared exposures at 15°C exhibited higher effect than 20°C.

SOD

The SOD activity showed significant ($P < 0.05$) inhibitions in discrete brain regions of offspring born to rats on exposure to individual stressors [Figure 1b]. GDM found to cause higher suppressions in SOD activity than cold-stressed subjects. In the coexposure group, cold-stress regimen at 15°C and 20°C

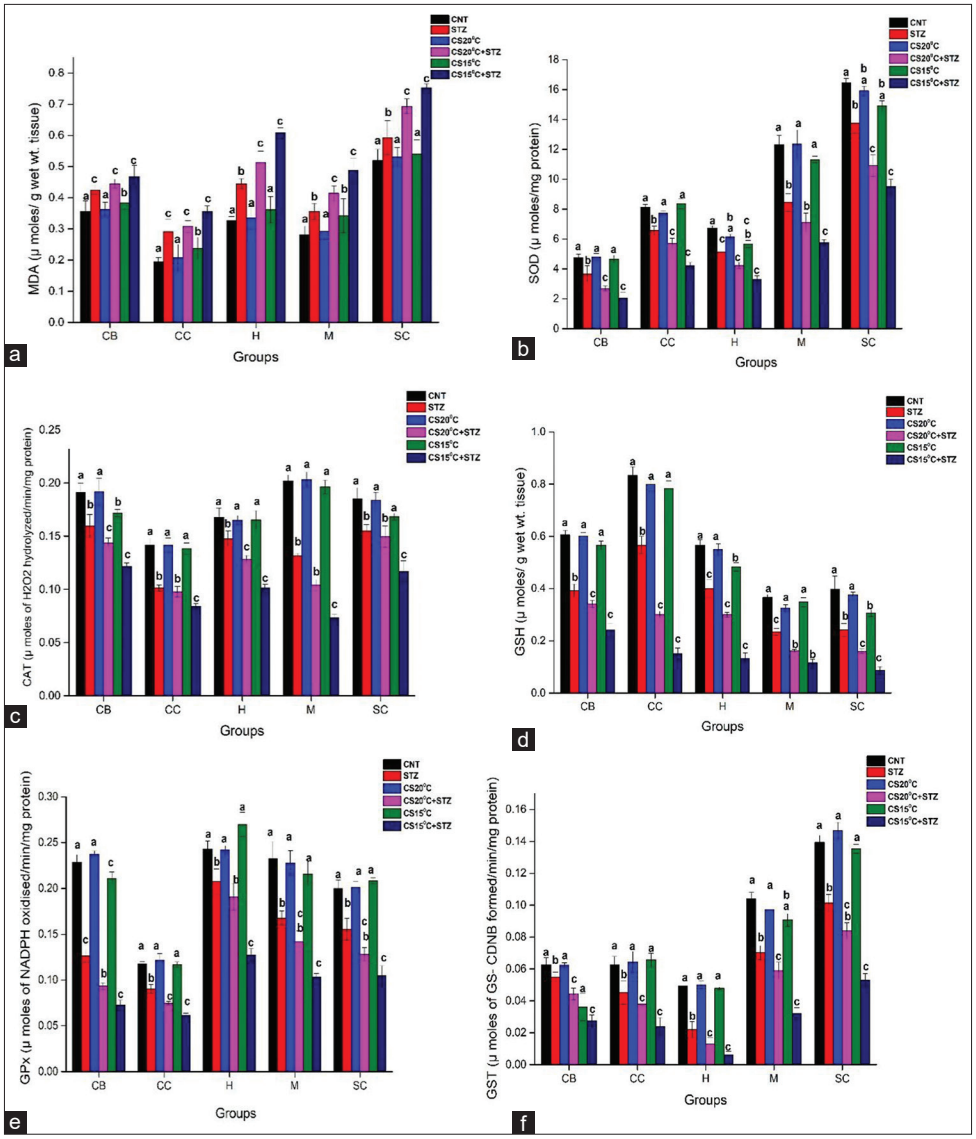

Figure 1: (a-f) Prenatal stressors induced oxidative changes in offspring brain regions: An interactive study of cold stress (CS-15 and 20°C) and gestational diabetes (streptozotocin induced). (a) Changes in MDA content; (b) changes in superoxide dismutase activity; (c) changes in catalase activity; (d) changes in glutathione levels; (e) changes in GPx activity; (f) changes in glutathione S-transferase activity. Values are mean ± SE of six rats in each group; Alphabets "a, b, c" are significantly different among experimental groups in the intergroup analysis. $P < 0.05$ as determined by one-way ANOVA followed by Dunnett's multiple comparison *post hoc* test. Discrete brain regions: CB: Cerebellum, CC: Cerebral cortex, H: Hippocampus, MO: Medulla oblongata, SC: Spinal cord

exhibited exacerbatory actions by suppressing higher SOD activity and affected brain regions were CC, medulla, and H, however exposures at 15°C demonstrated greater effect than 20°C.

Catalase

The data representing catalase (CAT) activity showed significant ($P < 0.05$) decrements in brain regions of offspring, wherein STZ administration during gestation caused higher suppressions in cortex and medulla while cold stressed subjects at 15°C exhibited moderate inhibitions in the CB and SC [Figure 1c]. An exacerbated effect witnessed in the coexposure group, wherein exposures at 15°C and 20°C found to cause higher suppressions in CAT activity especially in medulla, cortex, and H regions, furthermore, exposures at 15°C displayed higher reductions than 20°C.

GPx

A significant ($P < 0.05$) inhibition in GPX activity was evident in discrete brain regions of offspring born to rats on exposure to individual stressors such as GDM and cold stress (15 and 20°C). In rats, STZ administration during gestation caused higher suppressions in the CB, cortex, and medulla regions while exposures at 15°C exhibited moderate inhibitions in GPX activity in the CB and H (Figure 1d). An exacerbated effect was witnessed in the offspring of dual stressor group wherein gestational exposures at 15°C and 20°C found to cause higher inhibitions in the CB, medulla and, cortex

regions. Furthermore, exposures at 15°C exhibited higher destruction than 20°C.

GST

Significant ($P < 0.05$) inhibition in GST activity was observed in discrete brain regions of offspring born to rats on exposure to individual stressors such as GDM and cold stress, wherein STZ administration during gestation caused higher suppressions in H, medulla and cortex while cold stressed subjects (at both 15°C and 20°C) exhibited moderate inhibitions only in the medulla [Figure 1e]. An exacerbated effect was witnessed in the dual stressor group where exposures at 15 and 20°C caused higher suppressions in the H, medulla and cortex regions. Furthermore, exposures at 15°C found to cause higher suppression than 20°C.

Reduced GSH

The GSH levels found diminished in discrete brain regions of offspring born to dams on exposure to prenatal stress. Individual treatments caused reductions in GSH content, while in coexposure brought an exacerbated effect indicating higher suppressions [Figure 1f]. Among discreet brain regions, cortex, H, and CB exhibited high vulnerability. Likewise, exposures at 15°C found to cause a higher reduction than 20°C.

Gestational diabetes is sensitive and affects the development of the fetal brain. *In utero* studies on stress-induced changes in response to cold exposure in diabetic subjects are unknown. Whether the cold-stress, affects the development of brain is unknown.

DISCUSSION

In utero studies on stress-induced changes in response to cold exposure in diabetic subjects are unknown. To examine the effect, this study was conducted to assess the changes occurring in the level of oxidative stress indices in brain regions of rat offspring born as a consequential exposure to gestational diabetes (STZ induction) and cold stress (15 and 20°C). Interaction of cold-stress provoked higher degree oxidative stress in brain compartments as evidenced by a further decrease in antioxidant enzymes, namely, SOD, catalase, GPx, GST, and GSH as well as increase in the concentration of MDA. The findings of this study are promising and strengthen the recent findings of Fatima *et al*.[27] wherein exposure to chronic mild stressors in hit or miss manner induced marked behavioral disturbances in dams which led to the event of prenatal stress in rat offspring.[27] However, their findings were limited to prove how maternal psychological stress features a widespread effect on fetal outcomes through major physiological alteration within the antioxidant levels, neurogenesis, signaling molecules, and DNA damage. The findings of this study highlight the synergistic actions of stressors due to the increased generation of free radicals and cold stress interaction(s) found to cause exacerbatory actions by depleting antioxidant enzymes. Besides, the brain regions showed a distinct variation in the antioxidant enzyme activities in response to cold stress.

Using MDA as a marker, Huerta-Cervantes *et al*.[28] reported gestational diabetes triggered oxidative stress in the H and CC resulting in modification of cognitive behavior in offspring and our study results are partly corroborating with above findings. Besides, cold-stress found to cause exacerbated effect in augmenting higher MDA levels in GDM offspring and comparison of exposures exhibited a higher effect at 15°C [Figure 1a]. Conversely, marked inhibition in the activities of the SOD and catalase, especially in the cortex, H, and to varying degrees in other brain regions, was seen in rat offspring. These biochemical changes suggest vulnerability to oxidative stress in the brain and are region-specific. The curtailed activity of SOD observed in discrete brain regions [Figure 1b] could be due to its reduction or inhibition as a result of the amplified production of free radicals and that they can potentially end in cellular damage. Although catalase is not essential to some cell types under normal conditions, it is crucial role in the acquisition of tolerance to oxidative and nitrosative stress during the cellular adaptive response[29] is important. In this study, catalase activity reduced altogether in brain regions of offspring wherein GD caused higher suppressions in cortex and medulla while cold stressed subjects at 15°C exhibited moderate inhibitions in the CB and SC. An exacerbated modulation in CAT activity observed in coexposure groups, wherein exposures at 15°C and 20°C found to cause higher suppressions in medulla, cortex, and H regions, furthermore, exposures at 15°C displayed higher reductions than 20°C [Figure 1c].

The GSH antioxidant system has an important role in cellular defense against reactive free radicals and other oxidant species. Few studies reported stress-mediated alterations in the antioxidant system in the brain regions especially, the prefrontal cortex, and H that are most susceptible to the pathophysiology of stress.[29,30] In this study, the reductions observed in the level of GSH system comprising GSH, GSH-Px, and GST [Figure d-f] are probably due to an increased requirement of antioxidant defense to encounter the elevated peroxidative challenge. The data presented here support the impression that prenatal stress in the sort of an abnormal intrauterine milieu may cause long-lasting changes in the offspring of the subjects exposed to diabetes during the gestational period. A modulation in GPX and GST activities was observed in the offspring of dual stressor group wherein gestational exposures at 15°C and 20°C found to cause higher inhibitions in GPX activity in the CB, H, and medulla. Likewise, GST activity too was affected in the H, medulla, and cortex regions. These

variations in the levels of antioxidant enzymes make the tissues vulnerable to oxidative stress resulting in functional impairments.

From aforesaid observations, it is evident that prenatal stress has significant long-lasting sway on brain antioxidant status in offspring. In principle, prenatal stress relies on the potential role of hyperglycemia in the etiology of neurotoxicity through oxidative stress in diabetic subjects. Explicitly, in gestation, both the mother and therefore the fetus are hyperglycemic due to free transport of glucose through the placenta thereby the fetus is forced to extend its insulin production.[2] Additively, acute cold exposure increases glucose supply to the fetus, which stimulates further insulin secretion.[31] Thus, the fetus adapts to the changes to which the mother is exposed, if the exposures are chronic, the fetus develops all of its organs to organize for the external milieu and it could induce permanent changes in cellular structure, organ physiology, and metabolism.[27] Changes in the histology of placenta in gestational diabetic subjects on account of fetal hypoxia are reported by Augustine *et al*.[32] and their findings establish an association with a high risk of prenatal and postnatal complications. Further, the stage of gestation during which the strain applied is extremely important as prenatal stress shown to be harmful when applied during early gestation. In this study, the twin stressor paradigm applied from day-4 of gestation has effectively altered the antioxidant status in discrete brain regions [Figure a-f] leading to long-standing effects on the CNS which is to be relatively considered as sensitive to the oxidative insult(s), more so at the developing stages as it contains high levels of easily oxidizable fatty acids, low antioxidant defense system and uses the massive expanse of oxygen consequently rendering tissue susceptible to oxygen radicals.[33] Furthermore, the heterogeneity of developing brain(s) with different cell types and performance makes it more susceptible to multiple stressors due to the prevalence of excessive free radicals.[34] The results in this study have demonstrated the exacerbatory actions, wherein exposures at 15°C and 20°C found to cause higher suppressions in antioxidant enzymes especially in discrete regions, furthermore, exposures at 15°C displayed higher reduction than 20°C. Available data also suggest that the involvement of the HPA axis through its hormonal response (via cortisol), is responsible for critical neurodevelopmental deficits in offspring which is transduced due to gestational stress.[27,35] It can be inferred from above observations that cold stress at lower temperatures (15°C) produces chronic adrenergic overload (sympathetic activation) which will affect the development of the fetus,[9,10] and therefore the reduced capacity of antioxidants thereby higher vulnerability observed in discrete brain regions. Whether these changes are adaptive or compromise the capacity of the brain to deal with the dual stressor paradigm-induced oxidative stress that could lead to degenerative neurotoxic manifestations remain to be understood.

CONCLUSION

Prenatal stress has significant long-lasting sway and exaggerated postnatal development on brain antioxidant status in the offspring. The findings prove that cold stress is a crucial stimulus to a fetus during gestation and acts as a trigger of oxidative stress especially in diabetic subjects and can pose an adverse impact. Low temperatures exert exacerbated effects consequently functional deficits do arise in developing brain involving synergetic interactions that have deleterious effects on long-term development. More research is required to work out whether these effects are often replicated in other populations and whether or not they become larger with age.

ACKNOWLEDGMENTS

Rizwan Sharief is grateful to “Karnataka Directorate of Minorities Scholarship,” Bengaluru, India, for partial financial support and awarding research fellowship.

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How to cite this article: Sharief R, Mahaboob A, Basha PM. Consequential exposure to hypothermia in gestational diabetic rats induces oxidative changes in the brain of offspring. Int J Med Sci Public Health 2020;9(7):407-413.

Source of Support: Nil, **Conflicts of Interest:** None declared.